Chemical and molecular basis of the carcinogenicity of *Aristolochia* plants

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Abstract

The old herbal drug aristolochic acid (AA), derived from *Aristolochia* species has been associated with the development of a novel nephropathy, designated as aristolochic acid nephropathy (AAN), and human urothelial cancer. The major components of the plant extract AA are nitrophenanthrene carboxylic acids, which are genotoxic mutagens after metabolic activation. The major activation pathway involves reduction of the nitro group primarily catalysed by NAD(P)H:quinone oxidoreductase to an electrophilic cyclic *N*-acylnitreniumion that reacts preferentially with purine bases to form covalent DNA adducts. These specific AA-DNA adducts have been identified and detected in experimental animals exposed to AA or botanical products containing AA, and in urothelial tissues from AAN patients. In rodent tumours induced by AA the predominantly formed DNA adduct 7-(deoxyadenosin-N^6-yl)aristolactam I has been associated with the activation of *ras* oncogenes through a characteristic transversion mutation. Such A:T→T:A mutations have been identified in the *p53* gene of urothelial tumours of a patient with AAN and in several patients suffering from Balkan endemic nephropathy (BEN) along with specific AA-DNA adducts. This is a rare example of a human cancer causally linked to a distinct environmental exposure (use of a herbal product), where the carcinogenic process of initiation is well-established linking formation of carcinogen-specific exposure (specific DNA adduct formation) with the presence of characteristic human tumour mutations.

Abbreviations

AA, aristolochic acid; AAN, aristolochic acid nephropathy; BEN, Balkan endemic nephropathy; CHN, Chinese herbs nephropathy; CYP, cytochrome P450; dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I; dA-AAII, 7-(deoxyadenosin-N^6-yl)aristolactam II; NQO1, NAD(P)H:quinone oxidoreductase;

Introduction

This review discusses the molecular mechanism of the herbal product aristolochic acid (AA) leading to cancer in experimental animals and humans. These mechanistic investigations gathered from 1986 to present contributed that herbal remedies containing plant species of the genus *Aristolochia* were classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC) [1] and that the National Toxicology Program (NTP) considers AA as a substance known to be a human carcinogen [2].

AA, the principal extract of *Aristolochia* species is a mixture of structurally related nitrophenanthrene carboxylic acids, the major components being aristolochic acid I (AAI) and aristolochic acid II (AAII) (Figure 1). AA is found in plants of both the *Aristolochia* and *Asarum* genera of the family *Aristolochiaceae*. Herbal drugs derived from *Aristolochia* species have been used since antiquity in obstetrics and in the treatment of snake bite, festering wounds, and tumours, and they remain in use today, particularly in Chinese herbal medicine [1]. All parts of the plant are used for herbal remedies, and AA is present in roots, stems, leaves, and fruit. In the 1970s the anti-inflammatory properties of AA encouraged the development of pharmaceutical preparations in Germany until Mengs and coworkers showed that AA is a strong carcinogen in rats [3]. Subsequently, AA was found to be a genotoxic mutagen and all pharmaceutical preparations containing AA were withdrawn from the market in Germany and in many other countries. However, *Aristolochia* plants are still used in traditional medicine in some parts of the world [4].

So-called Chinese herbs nephropathy (CHN), a unique type of rapidly progressive renal fibrosis, was first reported in Belgian women who had consumed Chinese herbs as part of a wheight-loss regimen in 1991 [●5]. So far over 100 CHN cases have been identified in Belgium [4]. The observed nephrotoxicity has been traced to the ingestion of *Aristolochia fangchi* inadvertently included in the slimming pills [6].

Exposure to AA was demonstrated by the identification of specific AA-DNA adducts in urothelial tissue of these CHN patients using a highly sensitive detection method (^{32}P-postlabelling method) [●7-9]. Within a few years CHN patients developed a high risk of urothelial cancer; urothelial malignancy of the upper urinary tract arose in almost half of the patients [●●9]. According to the review by Debelle and colleagues [4] in 2008 CHN has been described in patients in other European (Germany, UK, France, Spain) and in Asian countries (China, Taiwan, Japan, Korea) and in the USA (about 170 cases), who had been exposed to *Aristolochia* species containing AA and had no relationship with the Belgian cohort. Therefore, it has been proposed to designate this novel interstitial nephropathy in which the unequivocal role of AA has been fully documented as aristolochic acid nephropathy (AAN) [10]. Since more and more AAN cases are reported world-wide and all are related to exposure to AA, it is of great concern that this form of nephropathy and associated urothelial cancers may occur more commonly in the future [4]. As a consequence products containing AA have been banned in many countries world-wide and consumers have been advised to discontinue immediately the use of any botanical products containing AA (Food and Drug Administration consumer advisory, 2001). However, despite warnings, a number of herbal products containing *Aristolochia* species continue to be advertised for sale on the internet [4].

The intention of this review is to summarize data on the genotoxic mechanism of AA carcinogenicity in animals and humans. The pathogenesis of the AA nephropathy has been reviewed recently by Debelle colleagues [4].

Carcinogenic mechanism of aristolochic acid in animals

Carcinogenicity in animals

The natural mixture AA is a strong carcinogen in rats [1, 3]. Main targets for AA-induced carcinogenicity were forestomach, kidney and urinary tract. AA is also a potent carcinogen in mice after oral treatment and in rabbits after intraperitoneal injections. Most studies administered a mixture of aristolochic acids I and II; however, similar carcinogenic effects were also observed with pure aristolochic acid I [11, 12]. Moreover, complete extracts from *Aristolochia* species (decoctions from *A. manshuriensis* and an aqueous extract of *A. fructus*), when administered orally to rats induced tumours of the forestomach and the kidney [13, 14]. Squamous-cell carcinomas were found in the forestomach of male rats treated with the weight-loss regimen of herbal ingredients that contained AA used in the Belgian slimming clinic [15] demonstating that the plant material ingested by AAN patients is able to induce tumour formation.

Metabolism of AA

The metabolism of AA has been studied in different species including man and has shown that products of nitroreduction, the corresponding aristolactams, are the major metabolites found in urine and faeces [16]. Other minor metabolites formed through *O*-demethylation and denitration have also been reported. In humans the aristolactams I and II were the only metabolites detected in urine although full metabolic profiles have not been reported. Phase II metabolites of aristolochic acids were found in the urine of rats and include *N*- and *O*-glucuronides, *O*-acetates and sulfate esters [17].

Metabolic activation of AA and DNA adduct formation

First hints on the mode of action of the rodent carcinogen AA came from studies in bacteria. AAI and AAII are direct mutagens in the *Salmonella* strains TA100 and TA1537 but were only weakly mutagenic in the strains lacking the classical bacterial nitroreductase [18]. Using genetically engineered YG strains Götzl and Schimmer [19] confirmed that only the nitro group is important for the mutagenic activity of AA in *Salmonella*. Nevertheless both AAs are only weak mutagens in the Ames assay (less than 1 revertant per nanomole) when compared to other nitroaromatic compounds [20].

A powerful tool of elucidating the pathway of activation of carcinogens is to identify and quantify the DNA adducts it forms, and to determine what factors modulate adduct formation. This approach was successfully applied and demonstrated that AA is a genotoxic mutagen after metabolic activation. For the detection of DNA adduct formation by AA *in vitro* and *in vivo* the ^{32}P-postlabelling assay was used, all AA-DNA adduct analyses reported were performed by this assay almost exclusively [21].

The ^{32}P-postlabelling assay is an ultrasensitve method for the detection and quantitation of carcinogen-DNA adducts [22]. It consists of 4 steps: (i) enzymatic digestion of DNA to nucleoside 3'-monophosphates; (ii) enrichment of the adduct fraction of the digest; (iii) 5'-labelling of the adducts with ^{32}P-orthophosphate; (iv) finally the labelled adducts are subjected to chromatographic separation, to generate a profile of adducts that can be visualized and quantified by measurement of their radioactive decay. The assay requires only microgram quantities of DNA and is capable of detecting adducts at frequencies as low as 1 in 10^9 normal nucleotides.

Using the ^{32}P-postlabelling assay characteristic maps on thin layer plates were obtained when DNA isolated from organs of animals treated by AA was analysed. These chromatograms

showed several distinctive adduct spots grouped as a specific spot pattern depending on the treatment. Treatment of rats by AAI resulted in the formation of three DNA adduct spots in forestomach DNA when analysed by the ^{32}P-postlabelling method with enrichment by nuclease P1 digestion. In contrast after AAII-treatment two AA-DNA adducts were detected and the mixture AA showed a combination of adduct patterns of the main components. Such a characteristic spot pattern can be used as a fingerprint left by AA on DNA to monitor exposure to animals and humans. Numerous studies using the ^{32}P-postlabelling method have shown that AA or the pure major components AAI or AAII form specific DNA adducts in several organs of treated rodents confirming that the chemical carcinogen AA acts by a genotoxic mechanism *in vivo* [1].

Three of the four AA-DNA adducts were identified by comparison with structurally identified synthetic adducts prepared by reductive activation of AAI and AAII as 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI), 7-(deoxyguanosin-N^2-yl)aristolactam I (dG-AAI) and 7-(deoxyadenosin-N^6-yl)aristolactam II (dA-AAII) [21] (Figure 2) and assigned to the adduct spots as shown in Figure 3. The dA-AAII adduct is formed from AAII but also from AAI through a demethoxylation reaction. A second deoxyguanosine adduct formed by reaction of AAII with deoxyguanosine 3'-monophosphate and DNA was tentatively assigned as 7-(deoxyguanosin-N^2-yl)aristolactam II (dG-AAII) [21]. These chemical structures indicate that a cyclic *N*-acylnitrenium ion with a delocalised positive charge (aristololactam-nitreniumion) as the ultimate electrophilic species binds preferentially to the exocyclic amino groups of purine nucleotides in DNA through the C-7 position of the phenanthrene ring (Figure 2). Recently the structures of the purine AA-DNA adducts were confirmed by mass spectrometry along with AA-adducts bound to the exocyclic amino group of cytosine [23]. These AA-deoxycytidine adducts formed in *in-vitro* reactions with reductively activated AAI and AAII and DNA exhibited the same imino characteristics as shown before by NMR for the deoxyadenosine AA-adducts. However, AA-deoxycytidine DNA adducts have never been detected in *in-vivo* studies. This preference of AA for reaction with the exocyclic amino group of DNA bases is unusual for nitroaromatic compounds since their ultimate carcinogenic species is a nitrenium ion whose major target site in DNA is the C-8 atom of guanine.

It is well known that conjugation reactions like acetylation catalysed by phase II enzymes are important in the metabolic activation of carcinogenic nitroaromatics and aromatic amines. Concerning the activation of AA phase II reactions do not seem to play a role. Instead the formation of a cyclic hydroxamic acid (*N*-hydroxylactam) favoured by the carboxy group in peri position to the nitro group represents a unique example for an intra-molecular

conjugation (acylation), which leads to the ultimate carcinogen. Recently the *N*-hydroxylactam was detected in the urine of AA-treated rats confirming its formation during AA metabolism [23].

The enzymatic activation of AA was extensively studied using DNA binding as a probe for metabolic activation and has been reviewed by Stiborova and co-workers [16]. The major activation pathway, reduction of the nitro group is catalysed by a number of cytosolic and microsomal enzymes, cytosolic NAD(P)H:quinone oxidoreductase (NQO1) being the most efficient [24]. In hepatic microsomes reductive activation of AA was attributed to cytochrome P450 (CYP) 1A1 and CYP1A2. Another AA-activating enzyme is prostaglandin H synthase (cyclooxygenase, COX), which is highly expressed in urothelial tissue. Both AAs when activated by these different enzymatic systems produced AA-specific DNA adduct patterns similar to that obtained *in vivo*, confirming that nitroreduction is the crucial step in the pathway of metabolic activation of AA to their ultimate DNA binding species. No oxidative activation of AA has been reported, yet. However, the aristolactams, the principal metabolites of AA (Figure 2) are activated oxidatively forming the same DNA adducts as AA after reductive activation. Although the fraction of adducts produced by the aristolactams contributing to total AA-DNA adduct formation has not been determined it seems not to be significant. In fact, Dong and co-workers [25] found only low amounts of dA-AAI and dG-AAI adducts (50 times lower than that observed with AAI and AAII), with the highest levels in the target tissue, renal pelvis, in Wistar rats treated with aristolactam I.

Mutations induced by AA

Protooncogenes have been identified as genetic targets that are involved in chemical carcinogenesis. In rodents many chemical carcinogens activate the *ras* protooncogene by a single point mutation in codons 12, 13 or 61. Likewise, AA-initiated carcinogenesis in rodents is associated with the activation of H-*ras* by a specific AT→TA transversion mutation in codon 61 (CAA). This mutation occurs exclusively at the first adenine of codon 61 in all forestomach and ear duct tumours of rats treated with AAI [11]. The same *ras* mutation was detected in tumours of mice [26] and in forestomach tissue of rats treated with the plant extract AA [27]. AT→TA transversions are also the predominant mutations detected in reporter genes in AA-treated transgenic mice and rats along with an increased mutation frequency in the target organ [28-30].

This selectivity of AA for mutations at adenine residues is consistent with the extensive formation of adenine adducts and a higher persistence of them in comparison to guanine

adducts in target organs [31]. Moreover, the translesional bypass of adenine adducts of AA (*e.g.* dA-AAI) observed in primed DNA replication reactions containing oligonucleotides with defined AA-DNA adducts placed at specific sites points to a mutagenic potential resulting from incorporation of adenine opposite the adduct during DNA replication [32], indicating that an AT→TA transversion would be the mutagenic consequence.

Our postulated mechanism for the carcinogenicity of AA in rats is summarized in Figure 4.

Carcinogenic mechanism of AA in humans

Aristolochic acid and urothelial cancer

The outbreak of AAN in 1993 was associated with the ingestion of Chinese herbal remedies prescribed by a single clinic; so far 128 patients with AAN have been identified in Belgium, mostly women, half of whom needed renal replacement therapy, mostly including renal transplantation [33]. In 1990 this clinic in Brussels began prescribing slimming pills consisting of Chinese herbal remedies intended to contain, in part, *Stephania tetrandra* (for its purported diuretic effects). However, it was shown that *S. tetrandra* (Han Fang-ji) was inadvertently replaced by *A. fangchi* (Guang Fang-ji) presumably because both plants are used in Chinese folk medicine under similar names, *Fangji* [4].

AA-DNA adducts were identified in all urothelial tissues available for analysis of Belgian AAN patients by ^{32}P-postlabelling (Figure 3A and 3B). By contrast, DNA of kidneys from several patients with other renal diseases was virtually free of DNA adducts (Figure 3C). The presence of AA-DNA adducts in kidney and ureter demonstrated unambiguously prior exposure of these women to AA contained in plant material from *A. fangchi* [●7, 8, ●●9, 34].

Within a few years AAN patients developed a high risk of urothelial cancer; urothelial malignancy of the upper urinary tract arose in almost half of the patients [●●9]. The cumulative dose of *A. fangchi* was a significant risk factor; patients with an intake of 200 g of herbs (the average herbal intake) had a 50% risk of developing cancer. More recently, it was found that even patients who do not display the characteristic histological features of AAN are also at risk of malignancy [35].

This clearly indicates that AA is not only a strong rodent carcinogen but also a potent human carcinogen. In the meantime urothelial carcinoma associated with high levels of AA-DNA adducts in the urothelial tissue have been reported outside the Belgian cohort, pointing to the direct carcinogenic potential of AA in AAN patients [36]. Moreover, the demonstration that in rabbits and in rats [4], AA given as single drug causes similar renal interstitial fibrosis as

well as urothelial tumours as observed in AAN patients removed any doubt on the causal role of AA in AAN and AAN-associated urothelial malignancy.

The potential role of AA-DNA adducts in AAN-associated urothelial cancer

AA-DNA adducts are not only suitable biomarker for exposure to AA, but also they seem to play a critical role in the carcinogenic process of AA. In renal and ureteral tissue of AAN patients three AA-specific DNA adducts, one major (dA-AAI) and two minor ones (dG-AAI and dA-AAII) were identified [●7-10, 34, 36] by the ^{32}P-postlabelling method exhibiting the characteristic adduct pattern by thin layer chromatography (Figure 3). Cochromatographic analyses using independent separation systems proofed that these are the same AA-DNA adducts detected previously in animals exposed to AA with levels ranging from approximately 0.1 to 50 adducts per 10^8 nucleotides. On the other hand no difference was found between the levels of AA-DNA adducts in AAN patients with urothelial cancer and tumour-free AAN patients [9]. This might be due to the fact that adduct formation is not linear with dose at the high amounts of AA that AAN patients had ingested.

Recently, Grollman and colleagues [●●37] identified AA-DNA adducts in renal tissue of an American woman with documented exposure to AA (around 2 adducts per 10^7 nucleotides) using ^{32}P-postlabelling along with an electrophoretic separation of the adducts (^{32}P-postlabelling/PAGE). More importantly, in this study the adenine adducts of AAI and AAII were identified by liquid chromatography electrospray ionization/multistage mass spectrometry (LC-ESI/MS/MS) in this individual confirming details of the adduct structures and removing any doubt that spots or bands detected in tissues of AAN patients by the ^{32}P-postlabelling method represent the DNA adducts shown in Figure 2.

The persistence of AA-DNA adducts in human tissue even many years after cessation of the slimming regimen is noteworthy [9]. The most prominent adduct found in all AAN patients analysed so far is the dA-AAI adduct. In AA-treated rats irrespective of the tissue analysed the dA-AAI adduct is also the predominant adduct. That only the dA-AAI adduct remains in urothelial tissues for an extensive period of time (up to 10 years) is consistent with its life-long persistence in target tissues in rats [31]. Both, the longer persistence and higher initial levels of the dA-AAI-adduct in urothelial tissue of AAN patients probably contributed to the relative abundance of this adduct.

More than 50% of all human tumours contain a mutation in *p53*. In AAN patients urothelial atypia were associated with the overexpression of the p53 protein [33], suggesting that *p53* is mutated in AAN-associated cancer. In deed, in one AAN patient from the UK available for

analysis a characteristic AT→TA transversion mutation typical for AA was found in *p53* (exon 5; codon 139 AAG) in urothelial tumour cells [●38]. It is noteworthy that the mutated base the first adenine has the same neighbouring bases in codon 138/139 (GCC AAG) of *p53* as in codon 61 (CAA) of H-*ras* suggesting a sequence-specific mechanism during mutation induction. AT→TA transversions also accounted for most of the AA-induced mutations in human *TP53* knock-in [Hupki] mouse fibroblasts [39, 40] and, interestingly, one of these was at the first adenine of codon 139 (AAG) identical to the mutation found in the AAN patient.
These mutations could trigger tumourigenesis in humans in the same way like mutations in codon 61 of H-*ras* trigger tumourigenesis by AA in rodents and indicate the molecular mechanism whereby AA causes urothelial cancer in humans (Figure 4).

Metabolic activation of AA in humans

The metabolic activation of AA in humans has been reviewed recently by Stiborova and colleagues [16] and is comparable to that found in rodents. Most of the activation of AA in human hepatic microsomes is mediated by CYP1A2 and, to a lower extent, by CYP1A1. In human renal microsomes NADPH:CYP reductase and prostaglandin H synthase (cyclooxygenase, COX) are active. The most efficient enzyme in the activation of AA in human hepatic and renal cytosols, is like in animals NQO1.
Around 1500-2000 patients may have been treated in the slimming clinic in Belgium and thus, exposed to AA [41]. Therefore the identified AAN cases in Belgium (128) thus represent about 5% of the exposed population. Besides differences in the amount of AA intake, differences in carcinogen activation could be the reason for this individual susceptibility. Indeed, in the human *NQO1* gene two polymorphisms have been found in the general population and associated with an increased risk for urothelial tumours [42]. Remarkably, the frequency of homozygous *NQO1*2* mutation varies across ethnic groups and was reported to be approximately 5% in Caucasians [43].

Aristolochic acid and Balkan endemic nephropathy-associated urothelial cancer

In recent years evidence has accumulated that Balkan endemic nephropathy (BEN) is an environmental disease, whose clinical and histopathological features are remarkably similar with AAN [44]. That dietary intake of AA may be responsible for BEN and its associated urothelial cancer is a theory that was first proposed in 1969 by Ivic and is fully consistent with the unique epidemiologic features of BEN. This hypothesis, however, did not receive widespread support at the time. However, more recently the same observation was made in

endemic regions of Croatia, reviving the old hypothesis that exposure to AA of individuals living in endemic areas could occur by dietary intake of bread derived from wheat grain which was contaminated with seeds of *A. clematitis* [45].

As earlier proposed by us experimental evidence such as the detection of AA-DNA adducts in BEN patients and the identification of AA-specific mutation spectra in tumours of BEN patients would establish a strong molecular link between AA and BEN [44]. Indeed, AA-DNA adducts have been found in two out of three renal tissues collected randomly from farmers with upper urinary tract malignancy from areas endemic for BEN, although these patients were not classified as clearly suffering from BEN [46]. Recently AT→TA transversions have also been reported in patients suffering from BEN along with AA-specific DNA adducts [●●37]. In this study mutations at AT pairs accounted for 89% (17/19) of all mutations, with the majority of these (15/17; 78%) being AT→TA transversions. Strikingly, several *TP53* mutations [codons 131 (3×), 209 (3×), 280, 291] found in the urothelial tumours from BEN patients were also found in immortalised cells derived from primary human *TP53* knock-in [Hupki] mouse fibroblasts exposed to AA [40]. The frequency and predominance of AT→TA transversions may be regarded as a mutational signature for human exposure to AA. AA-DNA adducts were detected in all BEN patients with levels of total adducts around 5 adducts per 10^7 nucleotides. Such adducts were not detected in renal tissue of patients with urothelial cancer who resided in a nonendemic region of Croatia.

Collectively, these results provide new evidence that AA is a risk factor for BEN and BEN-associated urothelial cancer and confirm the molecular mechanism of AA carcinogenicity shown in Figure 4. However, the role of other factors in the pathogenesis of BEN cannot yet be ruled out.

Conclusions

There is increasing evidence that the plant extract AA or/and its major components AAI and AAII are responsible for the carcinogenic effects observed in humans who ingested *Aristolochia* plants or herbal medicines prepared from these plants. This conclusion is based on sufficient evidence of carcinogenicity in humans and experimental animals, together with data on the molecular mechanism demonstrating AAs carcinogenic potential.

Despite the fact that AA-containing remedies have been banned in several countries, human exposure to AA might still occur by usage of traditional herbal remedies. Therefore herbal

medicines should be subjected to the same stringent scrutiny and controls as common drugs before their release on the market. Owing to the fact that AA is both a powerful nephrotoxin and a human carcinogen all botanical-containing products known or suspected of containing AA should be banned from the market worldwide.

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●● Provides evidence that the DNA adducts found in AAN patients by ^{32}P-postlabelling are derived from AA and that mutations typical for AA are found in BEN patients thereby establishing AA as a risk factor for BEN and BEN-associated urothelial cancer.

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Legends to Figure

Figure 1: Chemical structures of aristolochic acid I (AAI) and II (AAII).

Figure 2: Metabolic activation and DNA adduct formation of aristolochic acid I (AAI) and II (AAII); 7-(deoxyadenosin-N^6-yl)aristolactam I or II (dA-AAI or dA-AAII), 7-(deoxyguanosin-N^2-yl)aristolactam I or II (dG-AAI or dG-AAII).

Figure 3: Autoradiographic profiles of DNA adducts obtained from DNA of (A) kidney and (B) ureter of a patient with aristolochic acid nephropathy (AAN) using the nuclease P1 enrichment version of the ^{32}P-postlabelling assay.

Figure 4: Postulated mechanism for the carcinogenicity of aristolochic acid in rodents and humans. dA-AAI; 7-(deoxyadenosin-N^6-yl)aristolactam I. See text for details.

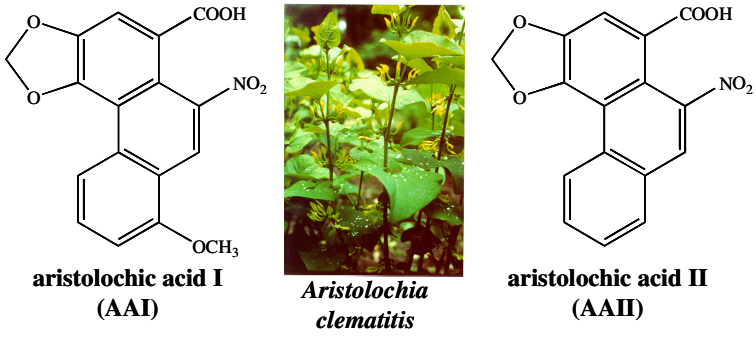

Figure 1

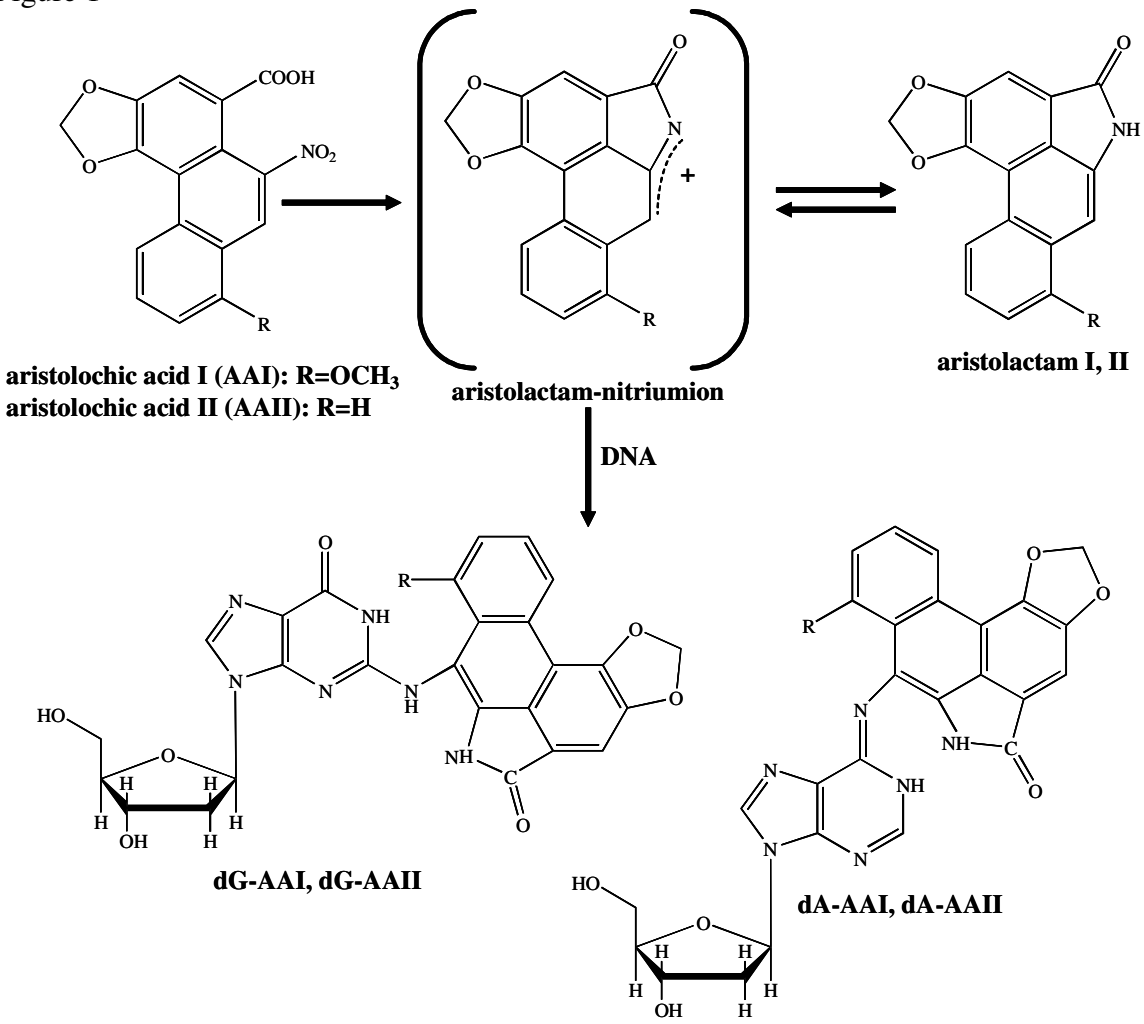
aristolochic acid I (AAI): R=OCH3
aristolochic acid II (AAII): R=H
aristolactam-nitriumion
aristolactam I, II
DNA
dG-AAI, dG-AAII
dA-AAI, dA-AAII

Figure 2

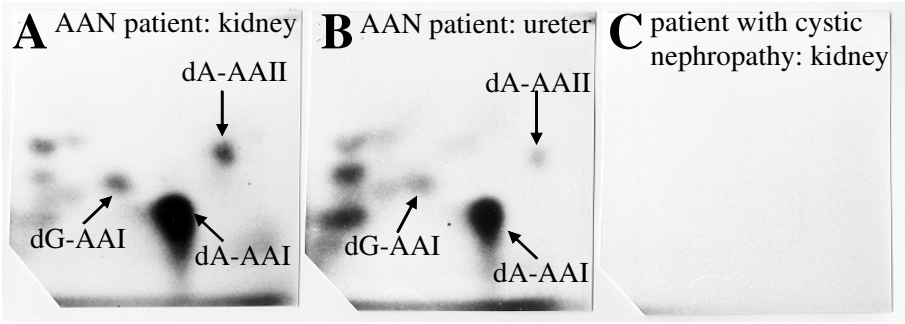
A
AAN patient: kidney
dA-AAII
dG-AAI
dA-AAI
B
AAN patient: ureter
dA-AAII
dG-AAI
dA-AAI
C
patient with cystic nephropathy: kidney

Figure 3

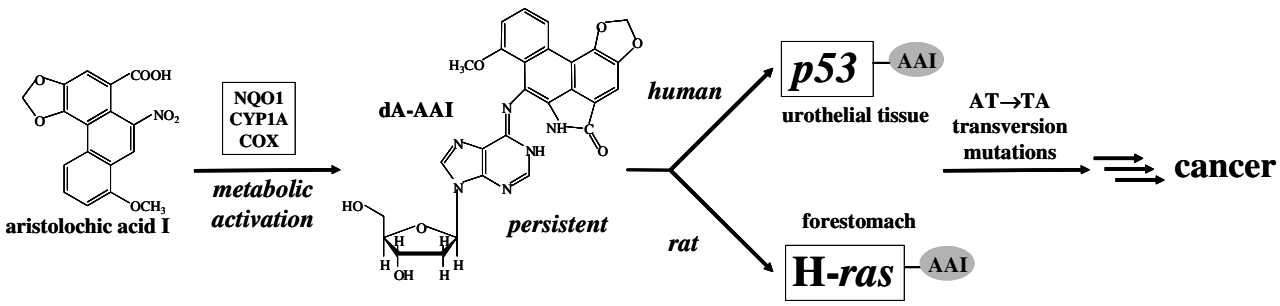
aristolochic acid I
NQO1
CYP1A
COX
metabolic
activation
dA-AAI
persistent
human
rat
p53
AAI
urothelial tissue
forestomach
H-ras
AAI
AT→TA
transversion
mutations
cancer

Figure 4